Loss of HtrA2/Omi protease activity induces mitonuclear imbalance and sarcopenia via differential regulation of mitochondrial biogenesis

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Research

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Abstract

Background

Cellular homeostasis requires tight coordination between nucleus and mitochondria, organelles that each possess their own genomes. Disrupted mitonuclear communication has been found to be implicated in many aging processes. However little is known about mitonuclear signaling regulator in sarcopenia which is a major contributor to the risk of poor health-related quality of life, disability and premature death in older people. HtrA2/Omi is a mitochondrial protease and play an important role in mitochondrial proteostasis. HtrA2 mnd2(-/-) mice harboring protease-deficient HtrA2/Omi Ser276Cys missense mutants exhibit premature aging phenotype. Additionally, HtrA2/Omi has been established as a signaling regulator in nervous system and tumors. We therefore asked whether HtrA2/Omi participates in mitonuclear signaling regulation in aging muscle.

Methods

Using motor functional, histological and molecular biological methods, we characterized the muscle phenotype of HtrA2 mnd2(-/-) mice. We employed bioinformatics analysis and identified HtrA2/Omi as a gene differentially associated with nDNA/mtDNA gene expression in sarcopenia. Further, we isolated the gastrocnemius muscle of HtrA2 mnd2(-/-) mice and determined expression of genes in UPR mt , mitohormesis, electron transport chain (ETC), and mitochondrial biogenesis.

Results

Here, we showed that HtrA2/Omi protease deficiency induced denervation-independent skeletal muscle degeneration. Despite of mitochondrial hypofunction, upregulation of UPR mt and mitohormesis related genes and elevated reactive oxygen species (ROS) production were not observed in HtrA2 mnd2(-/-) mice, contrary to previous assumptions that loss of protease activity of HtrA2/Omi would lead to mitochondrial dysfunction as a result of proteostasis disturbance and ROS burst. Instead, we showed that HtrA2/Omi protease deficiency results in different changes between the expression of nDNA and mtDNA encoded ETC subunits, which is in consistent with their transcription factors NRF-1/2 and coactivator PGC-1 α , suggesting that HtrA2/Omi protease activity may differentially regulate mitonuclear signaling via mitochondrial biogenesis in sarcopenia. Besides, Akt1 but not GSK3 β showed increased expression level in HtrA2 mnd2(-/-) muscles, indicating an involvement of Akt1 in PGC-1 α regulation response to HtrA2/Omi protease deficiency.

Conclusions

HtrA2/Omi protease deficiency induces mitonuclear imbalance and sarcopenia via differential regulation of mitochondrial biogenesis. The novel mechanistic insights may be of importance in developing new therapeutic strategies for sarcopenia.

Background

One of the most common changes to the body as it ages is the progressive loss of skeletal muscle mass and function that can progress to sarcopenia which is associated with significant morbidity and mortality and substantial healthcare costs [1, 2]. Mitochondria is critical to muscle function maintenance due to their roles in energy provision and redox homeostasis. Derangements in mitochondrial function have been advocated as major factors driving muscle degeneration [3]. Despite that mitochondria have their own genomes, the majority of mitochondrial proteins are encoded in nucleus. The expression of the mitochondrial proteome hence requires a coordinated dialogue between the two genomes, known as mitonuclear communication [4], to achieve a fine-tuned and functional mitochondrial population [5-7]. Accumulating evidences showed that mitonuclear communication may offer a more effective promotion of muscle healthy than mitochondria itself [6].

Various mitochondrial stress signals were sent in a retrograde fashion from mitochondria to the nucleus and initiate corresponding transcriptional adaptive response [5]. For instance, mitonuclear protein imbalance and the accumulated misfolded mitochondrial proteins activate the mitochondrial unfolded protein response (UPR^{mt}) to recover mitochondrial proteostasis in skeletal muscle [8]. Besides, mitochondrial stress substantially increases reactive oxygen species (ROS) in skeletal muscle, yet mediates an adaptation response named mitohormesis, and subsequently improves mitochondrial function. In fact, most of the identified mediators of retrograde signaling are proteotoxic signals, secondary metabolites and damaged mitochondrial components [9], it has not been fully elucidated whether exists mitochondrial localized proteins which directly transduce retrograde signals.

The high-temperature requirement protein A2 (HtrA2/Omi) [10] is a nuclear encoded serine protease that predominantly resides in mitochondrial intermembrane space (IMS) [11]. Traditionally, HtrA2/Omi has dual functions depend entirely on its subcellular localization. Upon stressful stimuli, HtrA2/Omi is released from mitochondria to cytosol and nucleus where it triggers caspase-dependent and independent apoptosis [12-18]. While under normal or moderate stress conditions (e.g. proteotoxic stress), HtrA2/Omi is restricted to the IMS where its serine protease activity is involved in the degradation of unwanted proteins to maintain mitochondrial proteostasis [19, 20]. Recent studies have demonstrated novel roles for HtrA2/Omi in many cellular processes such as cell proliferation, differentiation, autophagy, inflammation, immunomodulatory activity, mitochondrial dynamics and biogenesis [11, 21-27] via its protease activity, indicating an involvement of HtrA2/Omi in signals transduction.

HtrA2^{mnd2(-/-)} mice which harbor protease-deficient HtrA2/Omi Ser276Cys missense mutants suffer from neurodegeneration [27], motor abnormalities and premature death, suggesting a cytoprotective role for HtrA2/Omi protease activity [28]. HtrA2/Omi KO mice showed an accumulation of unfolded proteins in brain mitochondria, defects in the electron transport chain and increased ROS production [29]. Moreover, enhanced production of ROS upon loss of HtrA2/Omi was suggested to contribute to the activation of a mitochondrial stress response in brain [30]. Therefore, a prevailing hypothesis of HtrA2/Omi suggests that the loss of protease activity of HtrA2/Omi would lead to premature aging by destroying

mitochondrial proteostasis and promoting ROS production [29-32]. However, the upregulation of UPR^{mt}-related genes were not observed in HtrA2^{mnd2(-/-)} brain [30]. ROS levels were also found to be similar in macrophages of HtrA2^{mnd2(-/-)} mice compared to wild type (WT) [33]. Taken together, these facts raises the question of whether the cytoprotective effect of HtrA2/Omi might be achieved in a manner of mitonuclear signaling.

Herein, we set out to investigate the role of HtrA2/Omi protease activity in skeletal muscle using HtrA2^{mnd2(-/-)} mice. The data demonstrate denervation-independent sarcopenia induced by HtrA2/Omi protease deficiency. We also tested whether HtrA2/Omi protease deficiency impacts mitochondrial function by destroying mitochondrial proteostasis and promoting ROS production. Contrary to the expectation, we failed to observe neither the upregulation of UPR^{mt} and mitohormesis related genes, nor increased ROS level in HtrA2^{mnd2(-/-)} muscle. Thus we followed a data-driven approach using a sarcopenia gene expression microarray dataset, which provides an unbiased view of biological functions and genes expression correlated with HtrA2/Omi in sarcopenia without a previous defined hypothesis. The analysis were then biologically validated in HtrA2^{mnd2(-/-)} mice. The results demonstrated that loss of HtrA2/Omi protease activity results in mitonuclear imbalance and differential regulation of mitochondrial biogenesis genes, suggesting a role of HtrA2/Omi protease activity in mitonuclear coordination in sarcopenia.

Methods

Animals

The heterozygous mnd2 (HtrA2^{mnd2(+/-)}) mice (Stock Number:004608) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). HtrA2^{mnd2(+/-)} mice were interbred to generate populations containing all three genotypes (wild type, heterozygous, and homozygous). All animals were maintained under a 12-hour light/dark cycle with freely available food and water. 30 days homozygous male mice gastrocnemius muscles were used in this study. All procedures were carried out under the Guideline of National Institutes of Health, and approved by the Institutional animal Care and Use Committee of Jilin University.

Genotyping

Experimental offspring mice were identified by PCR-restriction enzyme analysis according to the protocol provided by JAX mice. Briefly, the Ser276Cys mutant allele of HtrA2 is detected by amplification of a 500 base pair (bp) fragment containing the exon-3 from nuclear DNA with the primers mnd2 (see supplementary table S1). Digestion genomic DNA with AluI produces a 244 bp product from the homozygous mice instead of 171 bp and 73 bp fragments from the wild-type. PCR of the digestion fragments was carried out and the products were separated on agarose gels and stained with GeneGreen (Tiangen, Beijing, China) [34].

Muscle contractile force measurements

Ex-vivo gastrocnemius muscle force measurements were performed as described previously [35]. Briefly, mice were anesthetized, and electrodes wires were placed on the sciatic nerve. The distal tendon of gastrocnemius muscle was mounted on a force transducer (Techman BL-420N, China) and kept in moisture with oxygenized (95% O₂ and 5% CO₂) Krebs–Henseleit solution (pH 7.6) at 30 °C. After optimizing the stimulation conditions and muscle length showing maximal isometric twitch tension, the muscle was allowed to rest for 5 min. Peak twitch tension were measured in single twitch (stimulus), and maximal tetanic tension was measured under series of stimuli. 30 s pause between stimuli was performed to avoid effects due to fatigue. After force measurements, animals were killed by cervical dislocation and muscles were dissected, weighted and stored for further experiments. Muscle contractile force was expressed in absolute and values normalized to physiological cross-sectional area (pCSA), which was calculated using the equation (1) [36]:

$$\text{pCSA (mm}^2\text{)} = \frac{M(\text{g}) \cdot \cos\theta}{\rho(\text{g/mm}^3) \cdot L_f(\text{mm})} \quad (1)$$

In the equation M is muscle mass, θ is pennation angle (default as zero), ρ is fresh muscle density (0.001056 g/mm³) [37-39], and L_f is fascicle length. The fascicle length of 6.6 mm was used in the calculation [38, 40, 41].

Histological analysis

The gastrocnemius muscles were fixed with 4% paraformaldehyde and embedded into paraffin. After dehydration and rehydration, 4 μ m coronal and sagittal sections were stained with haematoxylin and eosin (H&E). For Sirius red staining, the coronal sections was stained by Sirius red for 1 h, the other steps were the same as the H&E procedure. Finally, the morphology characteristics of the muscles were observed via microscope (ECLIPSE Ci-L, Nikon, Japan).

Muscle fibers morphological quantitative analysis

Morphological Quantitative Analysis of muscle fibers were performed with ImageJ software 1.52a (<http://rsb.info.nih.gov/ij/>) using images of H&E stained cross-sections. Briefly, Open-CSAM, an ImageJ macro supporting quantitative analysis of muscle fibers, was built in ImageJ according to a recently published work by Thibaut et al. [42]. H&E images were converted to grayscale in 8 bit with Photoshop software (Adobe Inc., San Jose, CA) before imported into Open-CSAM. Finally, the parameters of muscle fibers analyzed by Open-CSAM were exported in excel. Equations of Round score and Aspect Ratio were as follow (2-3):

$$Round = \frac{4\pi * A(\mu m^2)}{P^2(\mu m^2)} \quad (2)$$

$$Aspect\ ratio = \frac{F_{max}(\mu m)}{F_{min}(\mu m)} \quad (3)$$

In the equation (2), A is cross-section area (CSA) of muscle fiber, P is perimeter of muscle fiber, and in the equation (3) F is Feret's diameter. Round score and Aspect ratios near 1.0 describe circles.

Real-time quantitative PCR

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA). The RNA products was used to synthesize cDNA using Omniscript Reverse Transcription kit (TaqMan, Applied Biosystems, USA). qRT-PCR was performed to estimate mRNA levels using SYBR SuperMix (TransGen Biotech, CA). All reaction were done in duplicate on a Bio-Rad CFX96 Touch (Bio-Rad, Hercules, CA, USA). GAPDH was used as internal controls, relative expression level for each gene was calculated using $2^{-\Delta\Delta C_t}$ relative quantification method and normalized to the endogenous. All primer sequences are listed in Additional file 1: Table S1.

Mitochondrial copy numbers

Mitochondrial copy numbers were measured by absolute quantification RT-PCR as previously described [43]. Briefly, genomic DNA from gastrocnemius muscle tissues was isolated using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. Quantification of mtDNA copy number was performed in triplicates by qRT-PCR. mNADH1, mCYTB, mATP6 and mCOX2 were used as mtDNA markers, and β -globin nuclear intron was used as nDNA marker. Relative gene expression was normalized to that of the β -globin gene (ΔC_t) in each sample, and was normalized to the WT group. The primer sequences are listed in in Additional file 1: Table S1.

Measurement of ATP generation

Muscle tissue ATP content was measured using a bioluminescent assay kit (Beyotime, China) according to the manufacturer's instruction. Briefly, fresh tissue lysates were collected and then centrifuged at 12,000 g at 4°C for 10 min, the supernatant were then added into detection reagent. ATP content was measured using a multimode microplate reader (FLUOstar Omega, BMG Labtech, Germany), the relative luminescence unit (RLU) obtained were normalized to the protein concentration. The value of the WT control was set to 1.

Measurement of ROS generation

Fresh muscles were used to prepare a 10% (w/v) PBS homogenate. After centrifuging at 12,000 g for 10 min at 4 °C, the supernatant was collected and used to detect ROS and protein content. 90 µL of the supernatant and 10 µL of 1 mM DCFHDA (Beyotime, China) were added to each well of a 96-well plate. After incubating at 37 °C for 30 min in the incubator, the fluorescence was measured at 488 nm for excitation and 525 nm for emission using a multimode microplate reader (FLUOstar Omega, BMG Labtech, Germany). The results were calculated as the relative fluorescence unit (RFU)/µg protein. The value of the WT control was set to 1.

Western blot analysis

The gastrocnemius muscles were segregated and placed in liquid nitrogen for rapid freezing. Frozen samples were pulverized and lysed with 500 µl of the RIPA buffer (Beyotime, China). The lysates were ultrasonicated for 6*3 sec on ice and placed on ice for 45 min. Then the lysates were centrifuged at 4,500 g for 15 min at 4°C and the precipitate was discarded. Protein concentrations in the supernatants were determined using the Bradford reagent (Bio-Rad, Hercules, CA). The protein samples (10 µg) were resolved by 10%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membrane (Millipore, Billerica, MA, USA). Finally, immunodetection was performed using an enhanced chemiluminescence detection kit (DW101, TransGen Biotech, Beijing, China), then images were captured by Syngene Bio Imaging (Synoptics, Cambridge, UK). The following primary antibodies were used: HSP75 (10325-1-AP, Proteintech, USA), HSP60 (15282-1-AP, Proteintech, USA), HSP10 (sc-376313, Santa Cruz, USA), LONP1 (15440-1-AP, Proteintech, USA), CHOP (15204-1-AP, Proteintech, USA), ATF4 (sc-200, Santa Cruz, USA), Nrf2 (sc-13032, Santa Cruz, USA), Keap1 (10503-2-AP, Proteintech, USA), Foxo3 (10849-1-AP, Proteintech, USA), SOD1 (10269-1-AP, Proteintech, USA), SOD2 (A1340, Abclonal, USA), UCP2 (11081-1-AP, Proteintech, USA), GAPDH (AC002, Abclonal, USA).

Immunohistochemistry and staining

Fresh gastrocnemius muscles were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Paraffin sections were cut in section at 4 µm thickness, then deparaffinized and rehydrated before antigen retrieval. Sections were blocked with 10% bovine serum albumin (BSA) in TBS-Tween 20 (Sigma Aldrich) for 1 h at room temperature. Sections were incubated overnight at 4 °C with the respective following primary antibodies against PGC-1α (ab191838, Abcam, UK), NRF-1 (A14190, Abclonal, USA), NRF-2 (A8419, Abclonal, USA), VDAC1 (sc-390996, Santa Cruz, USA), AKT1 (10176-2-AP, Proteintech, USA) and GSK-3β (22104-1-AP, Proteintech, USA), and then incubated with secondary antibodies labelled with either Alexa Fluor 488 nm, FITC/Texas Red or biotin for 30 min at 37 °C. For IHC, slides were incubated with diaminobenzidine (DAB) for 5 min, and then counterstained with Gill's hematoxylin for 30 s. For IF, slides were incubated with Hoechst 33342 (1 µg/ml) for 5 min, then coverslipped with anti-fade media. Finally, images were captured on a fluorescent microscope (ECLIPSE Ci-L, Nikon, Japan).

Microarray data

The microarray datasets of GSE118825 was downloaded from Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>). These global gene expression profiles in gastrocnemius muscles contained a total of 54 specimens aged 6, 12, 18, 21, 24, and 27 months.

Construction of PPI network with an HtrA2 core

Protein-protein interaction (PPI) network were construct by uploaded HtrA2 and its neighbor genes of first three-layer in DEGs networks to the STRING database (<https://string-db.org/>). Then, the PPI network was further analyzed in Cytoscape software (www.cytoscape.org/). CytoHubba, a Cytoscape plug-in, sorts the genes by analyzing 12 parameters, including MCC, DMNC, MNC, DEGREE, EPC, BOTTLENECK, EcCentricity, CLOSENESS, RADIALITY, BETWEENNESS, STRESS and ClusteringCoefficient. We explore the genes sorting by 8 or more parameters as the hub genes with more essential in the functional network.

GSEA analysis

Gene Set Enrichment Analysis (GSEA) was performed using GSEA v4.0 software. Specimens were divided into low and high expression groups using HtrA2 expression quartile level as a cut-off points. In order to identify the potential function of HtrA2 in sarcopenia, GSEA was used to determine which gene sets were enriched in both groups. Number of permutation was set as 1000, and (c5.all.v6.2.symbols.gmt and c2.cp.kegg.v6.2.symbols.gmt, respectively) was used as reference gene-sets. A false discovery rate (FDR) q value <0.25 and nominal p value <0.05 was set as the significance cut-off threshold.

Correlation analysis

The genes of electron transport chain (ETC) subunits were obtained from the Molecular Signature Database (<http://software.broadinstitute.org/gsea/msigdb/>). Expression correlation among genes were calculated using “dplyr” and “tidyr” packages in R. The results were represented as Spearman's rank correlation coefficient. “ggplot2”, “circlize” and “ggstatsplot” package in R were used to draw bubbles plot, chordal graph and scatter plot, respectively.

Statistical analysis

All results are expressed as the mean $\pm$ SD. Two-tailed Student's t test was used to perform comparisons between two groups. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$. Comparisons between groups that do not follow a Gaussian distribution were performed via Mann Whitney U test. Statistical analyses were performed using GraphPad Prism software 8.0. Each replicate is 4 individual samples pooled.

Results

Overt symptoms of HtrA2^{mnd2}(-/-) mice

mnd2 was identified as a spontaneous and recessively inherited mutation that arose on a C57BL/6J background in 1990. Herein, wild type, heterozygous and homozygous *mnd2* mice (Figure 1a) were identified presymptomatically using the closely linked primers flanking microsatellite sequences in the C57BL/6J genome [34]. The earliest symptoms of HtrA2^{mnd2}(-/-) mice begin around 3 weeks of age, displayed as cessation of normal weight gain and progressive movement disorder. By 30 days of age, the mice become completely akinetic, and their weight was less than half that of WT and heterozygous littermates (Figure 1b). Details of the body weight, gastrocnemius muscle weight, and skeletal muscle mass index (SMI) are shown in table 1. The above data demonstrates that HtrA2/Omi protease deficiency results in decreased muscle mass.

Table. 1 Body and gastrocnemius muscle weight in *mnd2* mice

	WT	HtrA2 ^{mnd2} (+/-)	HtrA2 ^{mnd2} (-/-)	p value
Body Weight (g)	17.10±0.94	17.15±0.77	5.70±0.57	1.08E-05
Muscle Weight (g)	0.057±0.003	0.058±0.005	0.018±0.002	6.07E-06
SMI	0.331±0.007	0.337±0.012	0.314±0.011	3.96E-02
pCSA(cm ²)	0.081±0.004	0.083±0.006	0.026±0.002	4.00378E-07

The data represent average ± SD (n=4). Two-tailed unpaired Student's t test were used. Statistical significance: HtrA2^{mnd2}(+/-) vs. HtrA2^{mnd2}(-/-). Abbreviations: SMI, skeletal muscle mass index; pCSA, calculated physiological cross-sectional area.

Muscle performance

To understand whether muscle performance was affected, the strength of gastrocnemius muscle was measured in living mice of 30-day-old when only sporadic degeneration of motor neurons was observed in cervical spinal cord of HtrA2^{mnd2}(-/-) mice [44, 45]. The absolute single twitch and maximal tetanic force were reduced in HtrA2^{mnd2}(-/-) mice compared to WT controls (Figure 1c). The forces normalized for calculated physiological cross-sectional area (pCSA) of gastrocnemius muscle, were also decreased in HtrA2^{mnd2}(-/-) mice (Figure 1d-e), suggesting a poorer muscle performance in HtrA2/Omi protease-deficient mice.

Histological analysis of skeletal muscle

The gastrocnemius muscle revealed pathological changes in HtrA2^{mnd2}(-/-) mice. H&E stained cross sections (Figure 1f) of gastrocnemius muscle showed areas of myofibril fragmentation and rupture. Eosinophilic homogenous, hypercoagulated myofibers (hyalinization) indicating degeneration were

observed. Centralized nuclei (Figure 1f) and multinucleated fibers in HtrA2^{mnd2(-/-)} (Figure 1g) mice were also found which indicate skeletal muscle aging [46].

On H&E stained cross sections, an increase in inter-fiber space was observed in HtrA2^{mnd2(-/-)} mice compared with the WT control (Figure 1f). To exclude the possibility of sectioning artifact, sections were also stained by Sirius Red. Increasing collagen deposition was observed in the HtrA2^{mnd2(-/-)} muscle (Figure 1h), indicating the occurrence of muscle fibrosis, which is also an aging phenotype in skeletal muscle [46].

Muscle fibers morphological quantitative analysis

Primary anatomic changes in the aging muscle is decreased muscle mass and cross-sectional area [46]. In the present study, light microscopy images revealed cell thinning, CSA reduction in HtrA2^{mnd2(-/-)} muscle fibers (Figure 1f). To quantify how HtrA2/Omi protease deficiency affects muscle fiber CSA, we performed high throughput semi-automated analysis of CSA in cross-sections of H&E staining, 244 fibers in WT, 205 fibers in HtrA2^{mnd2(+/-)} and 154 fibers in HtrA2^{mnd2(-/-)} mice were analyzed (Figure 2a), all data is shown in Additional file 2: Table S2. The results showed a significantly smaller myofibers in HtrA2^{mnd2(-/-)} muscles compared with WT controls (Figure 2b). The frequency distribution of fiber CSA in HtrA2^{mnd2(-/-)} muscles showed a leftward shift toward smaller fiber size, with over twice the number of fibers with areas <1500 μm^2 compared with WT controls (Figure 2e). Skeletal muscle fibers were greatly deformed in HtrA2^{mnd2(-/-)} muscle (Figure 1f). Consistent with this observation, the Round score of HtrA2^{mnd2(-/-)} muscle fibers significantly decreased (Figure 2c), in addition, the Aspect Ratio of HtrA2^{mnd2(-/-)} muscle fibers significantly increased (Figure 2d), both indicating an increased irregularity degree of muscle fibers in HtrA2^{mnd2(-/-)} mice. The HtrA2^{mnd2(-/-)} muscle fibers showed a leftward shift in the frequency distribution of Round score (Figure 2f), while a rightward shift in Aspect Ratio (Figure 2g).

Fiber type analysis

HtrA2^{mnd2(-/-)} mice showed decreased expression in *Myh7* (encoding slow/type I MyHC), *Myh2* (encoding type IIa MyHC), *Myh1* (encoding type IIx MyHC) and *Myh4* (encoding type IIb MyHC) in gastrocnemius muscles (Figure 2h). Decline of both type I and II fiber numbers is an important change in aging muscles [46].

Overall, HtrA2^{mnd2(-/-)} muscle showed decreased muscle mass, impaired muscle performance, decreased cross-section area, presentation of morphological characteristics of aging and consistent decrease in fiber numbers of all types. Considering the diagnostic criteria of sarcopenia [2], these data support the notion that HtrA2^{mnd2(-/-)} mice exhibit sarcopenia.

Denervation-independent muscle degeneration

To determine whether the sarcopenia phenotype in HtrA2^{mnd2(-/-)} muscle is a result of neurodegeneration, denervation-sensitive genes were examined, there was no change in *MyoD* and *Myogenin* mRNA expression, and further, the mRNA expression level of acetylcholine receptor α (*AChR α*) and *AChR γ* significantly decreased in HtrA2^{mnd2(-/-)} mice, indicating that the sarcopenia phenotype observed in HtrA2^{mnd2(-/-)} muscle results from processes that originate in muscle rather than neurodegeneration.

HtrA2/Omi protease deficiency causes mitochondrial hypofunction

HtrA2 is a mitochondrial serine protease that is involved in the degradation of unwanted proteins to maintain mitochondrial proteostasis, to assess the effects of HtrA2/Omi protease deficiency on muscle mitochondria, we investigated the protein expression levels of mitochondrial membrane protein voltage-dependent anion channel 1 (VDAC1) which has been widely used as mitochondrial reference marker, as expected, VDAC1 protein expression level decreased significantly in HtrA2/Omi protease deficient muscles (Figure 3a-c). VDAC1 staining presented regularly striped arrangement in WT muscles which is an characteristic of functional and physiological muscles [47], while, in HtrA2^{mnd2(-/-)} muscles, diffused distribution of VDAC1 was observed, besides, “alopecia areata” of VDAC1 staining was also noticed (Figure 3a,b), which suggest abnormality in mitochondrial function in HtrA2^{mnd2(-/-)} muscles. Consistently, mtDNA copy number (Figure 3d) and ATP production (Figure 3e) also decreased significantly in HtrA2^{mnd2(-/-)} muscles. Interestingly, ROS level decreased but not increased in muscle of the homozygote *mnd2* mice (Figure 3f). ROS is a byproduct of mitochondrial respiration, generally speaking, mitochondrial dysfunction results in an increased ROS production, while mitochondrial hypofunction leads to the decline of ROS production as a result of low level of electron transportation [48, 49].

Absence of mitonuclear crosstalk in HtrA2^{mnd2(-/-)} muscles

A prevailing hypothesis of HtrA2/Omi suggests that HtrA2/Omi protease deficiency would destroy mitochondrial proteostasis and promoting ROS production [29, 30, 32]. To test this hypothesis, we detected the expression of several genes critical in UPR^{mt}, a cytoprotective response to maintain mitochondrial proteostasis. The results showed that the expression of heat-shock protein 75 (HSP75), HSP60, C-EBP homologous protein (CHOP) and mtDNAJ unchanged in mRNA or protein levels (Figure 4a,c-e), and further, the expression of activating transcription factor 5 (ATF5), mitochondrial lon peptidase 1 (LONP1), caseinolytic peptidase proteolytic subunit (CLPP) and caseinolytic peptidase chaperone subunit (CLPX) even decreased in HtrA2^{mnd2(-/-)} muscles (Figure 4a,c,e). Consistent with the results of ROS production, the expression of forkhead box O3 (Foxo3) and nuclear factor (erythroid-derived 2)-like 2 (Nrf2) also decreased in HtrA2^{mnd2(-/-)} muscles (Figure 4b,f). Foxo3 and Nrf2 have been recognized as key genes in mitohormesis which is an important adaptive response to ROS production [6]. Besides, both UPR^{mt} and mitohormesis are important mitonuclear communication mechanisms, which can be triggered by impaired mitochondrial function [6]. Collectively, the data suggests novel roles of HtrA2/Omi protease activity in mitochondrial function of skeletal muscle in addition of proteostasis maintenance.

HtrA2/Omi protease deficiency leads to mitonuclear imbalance

To predict biological functions of HtrA2/Omi in sarcopenia, analysis of GSEA, a powerful tool to infer the biological function, was performed using the sarcopenia microarray dataset. The results showed that biological processes associated with cellular respiration, mitochondrial ETC and oxidative phosphorylation were significantly enriched in HtrA2/Omi-low group (Figure 5a,b), all data is shown in Additional file 3: Table S3. These results suggest that HtrA2/Omi may participate in the process of sarcopenia via the regulation of ETC.

To further explore the regulation targets of HtrA2/Omi in ETC, 131 genes in KEGG_OXIDATIVE_PHOSPHORYLATION gene set, including all the core subunits of ETC complex I-V, were obtained from the Molecular Signature Database. Expression correlation analysis between these genes and HtrA2/Omi was conducted using the sarcopenia microarray dataset. Interestingly, the results showed that all the subunits that have strong negative expression correlation with HtrA2/Omi ($\gamma < -0.7$) are nDNA encoded, while, all the mtDNA encoded ETC subunits revealed either no correlation or strong positive correlation with HtrA2/Omi ($\gamma > 0.7$) (Figure 5c,d)(Additional file 4: Table S4). To validate the analysis results, we examined the mRNA expression levels of these genes. It turned out that most of the nDNA encoded ETC subunits showed decreased mRNA expression level in HtrA2^{mnd2(-/-)} muscles compared to WT controls (Figure 5e-i), while the mRNA expression levels of mtDNA encoded subunits either unchanged or increased in HtrA2^{mnd2(-/-)} muscles (Figure 5j). It should be noted that the sarcopenia groups in the microarray dataset showed increased expression level of HtrA2/Omi, which suggests that HtrA2/Omi may act as a compensatory regulator of mitochondrial function in sarcopenia. Taken together, the above data suggests a differential regulation of mtDNA/nDNA encoded ETC subunits by HtrA2/Omi protease activity.

HtrA2/Omi protease deficiency affects mitochondrial biogenesis

Peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) is one of the most important regulator in mitochondrial biogenesis, and participates in the regulation of ETC subunits through targeting transcription factors like nuclear respiratory factor 1 (NRF-1) and NRF-2, thus we investigated the correlation among HtrA2/Omi and these mitochondrial biogenesis genes using the sarcopenia microarray dataset. The results showed that HtrA2/Omi was negatively correlated with PGC-1 α , NRF-1 and its target ETC genes, while positively correlated with NRF-2 and its target ETC genes, and mtDNA encoding ETC genes (Figure 6a) (Additional file 4: Table S4). Consistently, The expression levels of PGC-1 α and NRF-1 decreased in HtrA2^{mnd2(-/-)} muscles (Figure 6b,d,e), PGC-1 α target genes glutathione peroxidase 1 (GPX1), superoxide dismutase 1/2 (SOD1/2), Catalase and uncoupling protein 2 (UCP2) also decreased in HtrA2^{mnd2(-/-)} muscles (Figure 6c,d), indicating a decline in PGC-1 α expression and function. While, there was an increased expression level of NRF-2 (Figure 6b,d,e). Besides, the NRF-2 target genes like COX8A, COX6A1 and COX17 revealed either no correlation or strong positive correlation with HtrA2/Omi ($\gamma > 0.7$) (Figure 5c,d). Consistently, the mRNA expression levels of COX8A, COX6A1 and

other NRF-2 target genes like SDHD and UQCRC1 also showed different changes from the other ETC subunits (Figure 5f-h) which are reported as NRF-1 target genes [50, 51]. It is noted that in mouse and rat, NRF-2 but not NRF-1 is responsible for mtDNA transcription and replication [50]. Overall the data suggests that HtrA2/Omi protease deficiency affects mitochondrial biogenesis, the differential expression of NRF-1/2 is probably the mechanism for mitonuclear imbalance observed in HtrA2^{mnd2(-/-)} muscles.

HtrA2/Omi protease deficiency may affect PGC-1 α via Akt1

As we found that PGC-1 α is decreased in HtrA2^{mnd2(-/-)} muscles, we wonder whether some factors that regulate PGC-1 α are changed. Therefore potential genes were screened through bioinformatics analysis using the sarcopenia microarray dataset. HtrA2/Omi was identified as the differentially expressed genes (DEGs), comparison between groups of 12 and 27 months was chosen for further analysis. PPI network of DEGs with an HtrA2/Omi core was constructed, among which eight genes were also identified as hub genes, including Tp53, Casp3, Akt1, Sod2, Vegfa, Myc, CS and Stat3 (Figure 7a). We noticed that among the hub genes Akt1 has been found to influence PGC-1 α abundance or activity through posttranslational modification, Akt1 phosphorylate PGC-1 α and promote its ubiquitination degradation [52]. Thus, Akt1 is probably responsible for the regulation of PGC-1 α by HtrA2/Omi.

In addition to Akt1, we screened for potential genes through literature review. Xu et al. reported that HtrA2/Omi promotes PGC-1 α degradation by cleaving glycogen synthase kinase 3 β (GSK3 β) in HtrA2^{mnd2(-/-)} brain. Therefore both Akt1 and GSK3 β were chosen for further evaluation. Firstly, expression correlation analysis was conducted using the sarcopenia microarray dataset. The results showed that Akt1 but not GSK3 β was negatively correlated with PGC-1 α , NRF-1 and its target ETC genes, while positively correlated with NRF-2 and its target ETC genes, and mtDNA encoding ETC genes (Figure 7b,c) (Additional file 4: Table S4). Secondly, the mRNA and protein expression of Akt1 and GSK3 β were also detected. Consistently, Akt1 but not GSK3 β showed increased expression level in HtrA2^{mnd2(-/-)} muscles, indicating an involvement of Akt1 in PGC-1 α regulation response to HtrA2/Omi protease deficiency.

Discussion

Disrupted mitonuclear communication is implicated in metabolic diseases, cancer, neurodegeneration, and other aging processes [6, 53-55]. While little is known about mitonuclear signaling regulator in sarcopenia. HtrA2/Omi is an IMS protease and play an important role in mitochondrial proteostasis [19, 20]. HtrA2^{mnd2(-/-)} mice harboring protease-deficient HtrA2/Omi Ser276Cys missense mutants exhibit premature aging phenotype [31]. Additionally, HtrA2/Omi has been established as a signaling regulator in nervous system and tumors [11, 21-27]. To better understand the role of HtrA2/Omi protease activity within skeletal muscle, we utilized HtrA2^{mnd2(-/-)} mice and found that loss of HtrA2/Omi protease activity induced denervation-independent skeletal muscle degeneration. Interestingly, upregulation of UPR^{mt} and mitohormesis related genes and elevated ROS production were not observed, contrary to previous assumptions that HtrA2/Omi protease deficiency would lead to mitochondrial dysfunction as a result of

proteostasis disturbance and ROS burst [31]. Instead, we found that loss of HtrA2/Omi protease activity results in mitonuclear imbalance and differential regulation of mitochondrial biogenesis genes, suggesting a role of HtrA2/Omi protease activity as a regulator of mitonuclear signaling in sarcopenia.

Sarcopenia observed in HtrA2^{mnd2(-/-)} mice is independent of neuromuscular degeneration

Our finding that the denervation-sensitive genes was unchanged in HtrA2^{mnd2(-/-)} muscle confirmed the prevailing view that physiological denervation is not responsible for the motor abnormalities in HtrA2^{mnd2(-/-)} mice. *mnd2* as its name “motoneuron disease 2” was originally characterized as a spinal muscular atrophy (SMA) because degenerating motoneurons were observed in late stages of the disease. However, Silvia et al. showed that there was no difference in mRNA level of denervation-sensitive gene AChR α between HtrA2^{mnd2(-/-)} and WT muscle, which distinguish SMAs from primary changes in the muscle, additionally morphological changes diagnostic for motoneuron disease were also not observed, indicating that *mnd2* is not a primary motor neuron disease [44]. Indeed, only sporadic degeneration of motor neurons was observed in cervical spinal cord of HtrA2^{mnd2(-/-)} mice after 30 days postnatal [44, 45]. In the present study, the HtrA2^{mnd2(-/-)} mice were sacrificed at 30 days postnatal. Considering the fact that mRNA levels, structure and weight of muscle did not significantly altered until 3-7 days after denervation [56-61], and that only 11% of specific force decrements are due to denervated fibers in rats [62], it suggests that the sarcopenia features observed in our study were not attributed to motor neurons degeneration. Consistent with our findings, Kang et al. showed that HtrA2^{mnd2(-/-)} mice were rescued from neurodegeneration by transgenic expression of HtrA2/Omi in the brain, and adult transgenic HtrA2^{mnd2(-/-)} mice still developed accelerated aging phenotypes including muscle atrophy, indicating a role of HtrA2/Omi protease activity in muscle aging. However, based on the observation that there was no significant difference in muscle fibers diameter of young transgenic rescued HtrA2^{mnd2(-/-)} mice compared with control mice, the authors concluded that the abnormalities observed in skeletal muscle of HtrA2^{mnd2(-/-)} mice are likely due to early onset neurodegeneration. It has been largely documented that organ crosstalk is important in physiological and metabolic processes. Therefore, it cannot exclude that the normal phenotype observed in skeletal muscle of transgenic rescued HtrA2^{mnd2(-/-)} mice is a result of the function of rescued brain.

HtrA2^{mnd2(-/-)} mice display a progressive striatal neurodegeneration with parkinsonian features [30]. Considering the motor control dysfunction and fatigability caused by PD, we detected muscle specific force production via ex-vivo gastrocnemius force measurements, to evaluate the degeneration in skeletal muscle. Neuronal degeneration in the striatum results in motor abnormalities as a consequence of failure of inhibitory inputs. Thus an ex-vivo muscle force test should be more suitable to reflect the function of skeletal muscle itself.

The prevalence of sarcopenia in the population aged 60 years or older ranged from 5% to 50% across studies [46, 63], while prevalence of sarcopenia in PD ranged from 6.6% to 55.8% [64-66]. A cross-sectional study showed that PD patients had a higher prevalence of sarcopenia (17.2% vs. 10.3%), but without significance (P adjusted=0.340), it also exhibited relatively preserved skeletal muscle mass in PD [67]. Similarly, another cross-sectional study conducted by Michela et al. concluded that sarcopenia due to a significant sparing of muscle mass, is an infrequent condition in PD, and likely to play a minor role in disability [64]. Loureiro et al. did not observed an increased prevalence of sarcopenia in PD [68]. Although the significant association of the motor part of PD with sarcopenia was also reported, it was suggested that there may be common underlying causes [65, 69-72]. Considering that HtrA2^{mnd2(-/-)} mice exhibited both PD and sarcopenia phenotypes, we proposed that sarcopenia in HtrA2^{mnd2(-/-)} mice is more likely a consequence of HtrA2/Omi protease deficiency than PD.

HtrA2/Omi may participate as an intermediate in mitonuclear cross talk.

A prevailing hypothesis of HtrA2/Omi suggests that the loss of protease activity of HtrA2/Omi would lead to premature aging [31] by destroying mitochondrial proteostasis and promoting ROS production [29, 30, 32]. Interestingly, we observed no change in neither expression level of UPR^{mt} genes nor ROS production in HtrA2^{mnd2(-/-)} mice. Similarly to our results, in a study by Moiso et al., CHOP and ATF3 was not upregulated in skeletal muscle or other non-neuronal tissues of HtrA2/Omi KO mice. Although CHOP expression was upregulated in brain, neither HSP60 nor CLPP mRNA were differentially expressed, suggesting that CHOP induction fails to induce any mitochondria protective genes in the brain of HtrA2/Omi KO mice [30]. It is noted that HtrA2/Omi is an IMS protease, Luena et al reported that IMS stress may activate a distinct UPR^{mt} in MCF7 breast cancer cell line by triggering estrogen receptor (ER) activity, which further upregulated the transcription of NRF-1 [73]. However, the transcription of NRF-1 was found to be downregulated in our study. Our results demonstrated that loss of HtrA2/Omi protease activity lead to mitochondrial dysfunction and skeletal muscle premature aging without activation of UPR^{mt}.

Rodrigue et al reported that ROS levels were similar in macrophages of HtrA2^{mnd2(-/-)} mice compared to WT [33], which is in agreement with our results. Conversely, three independent studies showed significantly elevated production of ROS in HtrA2/Omi KO mouse embryonic fibroblasts (MEFs) [30, 74, 75]. It was different from our results, as the authors employed HtrA2/Omi KO cells while we based our investigation on Ser276Cys missense mutated protease-deficient mice. Nicole et al. concluded in their study that regulation of mitochondrial morphology appears to depend on HtrA2/Omi protease activity, whereas mitochondrial ROS production could be related to another function of HtrA2/Omi [74]. Indeed, ROS is a by-product of OXPHOS, a normal mitochondrial function is the basis of ROS generation in cells. This may explain the decreased ROS level observed in our study as we observed that mitochondrial biogenesis genes as well as OXPHOS genes decreased significantly in HtrA2^{mnd2(-/-)} muscle. For further

validation, we found no change in mRNA levels of Foxo3, Keap1 and Nrf2 which have been recognized as mitohormesis related genes [5, 76]. Mitohormesis is a process in which low, non-cytotoxic concentration of ROS promotes mitochondrial homeostasis. Accumulating evidences revealed that other than a harmful redox product, ROS act as a mediator of skeletal muscle adaptation and associate with improved mitochondrial function [48, 77-79]. Meanwhile, mitochondrial-targeted antioxidant prevented the increase in mitochondrial biogenesis induced by caloric restriction (CR), and physical exercise [79-81]. Thus this unresponsive levels of ROS observed in our study may be another important cause of sarcopenia in HtrA2^{mnd2(-/-)} mice.

The disturbed balance between nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) encoded OXPHOS subunits, a state termed mitonuclear protein imbalance has been found to be associated with decreased mitochondrial respiration and UPR^{mt} activation [82, 83]. In our study, this imbalance was identified through GSEA and expression correlation analysis in which HtrA2/Omi showed strong negatively correlation with nDNA encoded ETC subunits, while no correlation or positively correlation with mtDNA encoded ETC subunits. It was further validated in HtrA2^{mnd2(-/-)} muscle, which showed different changes between the expression of nDNA and mtDNA encoded ETC subunits. Xu et al. showed that in brain of HtrA2^{mnd2(-/-)} mice both nDNA and mtDNA encoded ETC subunits decreased in mRNA level [27], this difference compared with our results in skeletal muscle may be due to tissue heterogeneity.

The present study reveals that loss of HtrA2/Omi protease activity per se was capable to induce mitonuclear imbalance. Paradoxically this imbalance in skeletal muscle of HtrA2^{mnd2(-/-)} mice failed to activate UPR^{mt}. We also noticed that different from other ETC complex, most of the subunits in ETC complex-II showed no change in mRNA expression in HtrA2^{mnd2(-/-)} mice. Complex-II is the only ETC complex consisting solely of nDNA encoded proteins, therefore does not require a balanced production of proteins from the nDNA and mtDNA.

Collectively, our findings suggest a role of HtrA2/Omi protease activity in mitonuclear signaling other than proteostasis maintenance. It should be noted that the present study only evaluated the mRNA levels, which may lead to a different conclusion, thus emphasizing the need for further investigation focusing on protein levels of ETC subunits.

PGC-1α may mediate the signaling function of HtrA2

Nucleus regulates mitochondrial adaptations by means of anterograde signaling which is mainly mediated by PGC-1α, a master regulator of nuclear-encoded mitochondrial genes (NEMGs) [4]. PGC-1α has been considered as the master regulator of mitochondrial biogenesis by targeting two key transcription factors, NRF1/2, which then activate nuclear genes encoding the OXPHOS subunits [84, 85]. We showed that the expression of PGC-1 and its target antioxidant genes decreased significantly in HtrA2^{mnd2(-/-)} muscle, indicating that HtrA2/Omi protease deficiency lead to impaired expression and cotranscriptional function of PGC-1α. Interestingly, NRF-1, NRF-2 and their target nDNA encoded ETC

subunit showed different changes in mRNA expression levels. Additionally, in mouse and rat, NRF-2 but not NRF-1 is responsible for mtDNA transcription and replication [50]. Collectively, PGC-1 α may be a downstream target of HtrA2/Omi and mediate the mitonuclear signaling function of HtrA2/Omi. The differential expression of NRF-1/2 is probably the mechanism for mitonuclear imbalance observed in HtrA2^{mnd2(-/-)} muscle, which call for further investigation.

Limitations

Our results leave open the possibility that some of the alterations in HtrA2^{mnd2(-/-)} muscle are secondary to effects in satellite cell. The effect of HtrA2/Omi protease deficiency on the regenerative capacity of satellite cell will be an important focus of future work. Another limitation of our study was that fascicle length coefficient of 0.66 was used for all genotypes in our study, however HtrA2^{mnd2(-/-)} mice may have a greater fascicle length due to their sarcopenia phenotype [86, 87], nevertheless, this would not change the significance of the results. Finally, no measures of fiber-type composition is presented and would be required to further elucidate the mechanisms underlying the sarcopenia phenotype in HtrA2^{mnd2(-/-)} muscle.

Conclusion

Our study focused on the role of HtrA2/Omi protease activity within skeletal muscle. We find that HtrA2/Omi protease deficiency induced denervation-independent skeletal muscle degeneration. Loss of HtrA2/Omi protease activity failed to induced UPRmt and mitohormesis related genes in spite of mitochondrial hypofunction. Instead, we showed that HtrA2/Omi protease deficiency results in different changes between the expression of nDNA and mtDNA encoded ETC subunits, which is in consistent with their transcription factors NRF-1/2 and coactivator PGC-1 α , suggesting that HtrA2/Omi protease deficiency induces mitonuclear imbalance and sarcopenia via differential regulation of mitochondrial biogenesis. The novel mechanistic insights may be of importance in developing new therapeutic strategies for sarcopenia.

Abbreviations

UPRmt: mitochondrial unfolded protein response; ROS: reactive oxygen species; HtrA2/Omi: high-temperature requirement protein A2; IMS: mitochondrial intermembrane space; pCSA: physiological cross-sectional area; RLU: luminescence unit; RFU: relative fluorescence unit; PPI: Protein-protein interaction; GSEA: Gene Set Enrichment Analysis; ETC: electron transport chain; SMI: skeletal muscle mass index; SMA: spinal muscular atrophy; AChR: acetylcholine receptor; VDAC1: voltage-dependent anion channel 1; glycogen synthase kinase 3 β (GSK3 β); HSP: heat-shock protein; CHOP: C-EBP homologous protein; ATF5: activating transcription factor 5; LONP1: mitochondrial lon peptidase 1; CLPP: caseinolytic peptidase proteolytic subunit; CLPX: caseinolytic peptidase chaperone subunit; Foxo3: Forkhead box O3; Nrf2: nuclear factor (erythroid-derived 2)-like 2; PGC-1 α : peroxisome proliferator-activated receptor γ coactivator

1 α ; NRF-1: nuclear respiratory factor 1; GPX1: glutathione peroxidase 1; SOD1: superoxide dismutase 1; UCP2: Catalase and uncoupling protein 2; GSK3 β : glycogen synthase kinase 3 β

Declarations

Author Contributions

H.Z., J.W., and L.S. designed, planned and coordinated the study, interpreted the data, and wrote the manuscript. D.Y., W.G., and H.S. performed the experiments. J.T. carried out data analysis and prepared figures. J.T. and S.Y. helped with manuscript and data review. All authors read and approved the final version of the manuscript. HSP75: heat-shock protein 75;

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Availability of data and materials

All data generated and analyzed during the study are available from the corresponding author on a reasonable request.

Ethics approval

All procedures were carried out under the Guideline of National Institutes of Health, and approved by the Institutional animal Care and Use Committee of Jilin University.

Consent for publication

All authors have read the final version of the manuscript and consented to its submission to Skeletal Muscle.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Sarcopenia phenotype observed in *HtrA2mnd2*(-/-) mice. (a) 30-day-old *HtrA2mnd2*(-/-) mice with genotype of WT (left), *HtrA2mnd2*(+/-) (middle) and *HtrA2mnd2*(-/-) (right). (b) Effect of the *HtrA2/Omi* Ser276Cys mutant on mouse body weight. Each point is the mean from four animals with standard deviations. *HtrA2mnd2*(-/-) mice do not gain weight after 20-day-old, and are significantly lighter than mice of the other two genotypes. (c-e) Ex-vivo muscle force measurements performed on gastrocnemius muscles. Loss of *HtrA2/Omi* protease activity leads to a significant decrease in both absolute (c) and pCSA normalized (d,e) force generated during single twitch or tetanic contraction. (f) Haematoxyline and eosin stain of cross-sections from gastrocnemius muscle of three genotypes. Arrowhead indicate

myofibers with hyalinization, characterized by eosinophilic homogenous and hypercoagulated fibers. Asterisks (*) indicate clear fat filled spaces at the inter-fiber space. Angular fibers can also be seen. Centrally localized nuclei (arrow) and ragged fibers were also found in HtrA2mnd2(-/-) muscles. (g) Haematoxyline staining showed multinucleated fibers in HtrA2mnd2(-/-) muscles. (h) Sirius Red staining. Collagen was stained in red, inter-fiber space showed increased deposition of collagen indicating muscle fibrosis. Slight fibrosis was also noticed in WT and HtrA2mnd2(+/-) mice. The data represent average $\pm$ SD (n=4). Two-tailed unpaired Student's t test were used. Statistical significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Scale bar, 50 μ m. Abbreviations: pCSA, calculated physiological cross-sectional area.

Figure 3

Sarcopenia phenotype observed in HtrA2^{mnd2}(-/-) gastrocnemius muscle. (a) High throughput semi-automated analysis of fiber CSA. 244 fibers in WT, 205 fibers in HtrA2^{mnd2}(+/-) and 154 fibers in HtrA2^{mnd2}(-/-) mice were recognized and analyzed. (b-d) Compared with WT controls, HtrA2^{mnd2}(-/-) muscles showed smaller fiber CSA and Round score, while larger Aspect Ratio. (e-f) Compared with WT controls, the frequency distribution of fiber CSA and Round score in HtrA2^{mnd2}(-/-) muscles showed a

leftward shift, while that of Aspect Ratio showed a rightward shift. (h) qRT-PCR analysis of Myh 1, 2, 4 and 7 gene expression in gastrocnemius muscle from mice of three genotypes (n=4). (i) qRT-PCR estimates of mRNA abundance of AChR α , AChR γ , MyoD and Myogenin in gastrocnemius muscles from mice of three genotypes. All estimates (n=4) are differences in mRNA relative to WT controls. In (b-d), Mann Whitney U test were used. In (h,i), two-tailed unpaired Student's t test were used. Statistical significance: *p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 0.001.

Figure 5

HtrA2/Omi protease deficiency causes mitochondrial hypofunction. (a) Immuno-histochemical staining of VDAC1 in gastrocnemius muscles. VDAC1 staining presents regularly striped arrangement in WT muscles (arrow). In HtrA2mnd2(-/-) muscles, diffused distribution and “alopecia areata” of VDAC1 staining are observed (arrowhead). (b) Immunofluorescence staining for VDAC1 in gastrocnemius muscles. Striped arrangement of VDAC1 is observed in WT controls but not HtrA2mnd2(-/-) mice (arrow). (c) Western blot analysis of VDAC1 and GAPDH expression. (d) qRT-PCR assay of COX2 was performed showing the relative mtDNA copy number (n=4). (e) Gastrocnemius muscle ATP contents were determined using ATP assay kit. Relative luminescence unit (RLU) were normalized to the protein abundance. Data is shown as a fold change compared to the mean value of WT controls which is ascribed an arbitrary value of 1 (n=4). (f) The generation of ROS was detected by using the ROS-sensitive probe DCFHDA. Relative fluorescence units (RLU) was normalized and shown in the same way as ATP contents (n=4). Two-tailed unpaired Student's t test were used. Statistical significance: *p $\leq$ 0.05; **p $\leq$ 0.01; ***p $\leq$ 0.001. Scale bar, 50 μ m. Abbreviations: DCFHDA, 2',7'-dichlorodihydrofluorescein diacetate.

Figure 7

Mitochondrial crosstalk associated genes are downregulated in HtrA2mnd2(-/-) muscles. Western blot analysis of (a) UPRmt and (b) mitohormesis associated proteins. (c-f) qRT-PCR assay of UPRmt and mitohormesis associated genes was performed (n=4). Two-tailed unpaired Student's t test were used. Statistical significance: *p≤0.05; **p≤0.01; ***p≤0.001.

Figure 9

HtrA2/Omi protease deficiency leads to mitonuclear imbalance. (a,b) GSEA of mitochondrial related GO or KEGG terms according to HtrA2/Omi expression level in the sarcopenia microarray dataset. (c) Expression correlation analysis between ETC subunits (complex I-V) and HtrA2/Omi. mtDNA encoded subunits are shown in the boxes. The bar length of each subunit represents absolute value of Spearman's rank correlation coefficient, and the bar color represents p value. (d) Chordal graph is drawn to display the correlation pattern between ETC subunits and HtrA2/Omi. Compartments represent ETC complex types, their subunits are noted along the outer margin, and their encoding genome are noted along the inner margin. Average Spearman's expression correlation coefficient between ETC subunits and HtrA2/Omi is used. The color of each box represents the value of correlation coefficient. (e-j) mRNA expression levels for part of the ETC subunits (complex I-V) in WT, HtrA2mnd2(+/-) and HtrA2mnd2(-/-) gastrocnemius muscle (n=4) were assessed by qRT-PCR. Two-tailed unpaired Student's t test were used. Statistical significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Abbreviations: FDR, false discovery rate; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Figure 11

HtrA2/Omi protease deficiency affects mitochondrial biogenesis. (a) Expression correlation heatmap among HtrA2/Omi and mitochondrial biogenesis genes. The value is presented as spearman's correlation coefficient. Different correlation patterns between NRF-1 and NRF-2 target genes are noticed. (b,c) qRT-PCR assay of mitochondrial biogenesis genes (b) and PGC-1 α target genes (c). (d) Western blot analysis of mitochondrial biogenesis genes and PGC-1 α target genes. (e) Immunohistochemical and Immunofluorescence staining of PGC-1 α , NRF-1/2 in gastrocnemius muscles. Two-tailed unpaired Student's t test were used. Statistical significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Scale bar, 50 μm .

Figure 13

HtrA2/Omi protease deficiency may affect PGC-1 α via Akt1. (a) PPI network of DEGs with an HtrA2/Omi core was constructed. (b) Heatmap of expression correlation among Akt1, GSK3 β and mitochondrial biogenesis genes. The value is presented as spearman's correlation coefficient. Akt1 but not GSK3 β is negatively correlated with PGC-1 α . (c) Expression correlation analysis is displayed as scatter plots. (d,e) qRT-PCR assay of Akt1 and GSK3 β expression (n=4). (f) Immunohistochemical staining of Akt1 and

GSK3 β in gastrocnemius muscles. Two-tailed unpaired Student's t test were used. Statistical significance: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. Scale bar, 50 μm .

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